Repair of intervertebral disc degeneration using KLD-12/TGF-β1 fiber gel combined with BMSCs in a rabbit model

Xiangchen Li, Hui Shao, Bin Wu, Yang Yu, Yan Du, Shaozheng Liu, Jianhua Sun

Department of Orthopedics, The First Affiliated Hospital, Shihezi University College of Medicine, Shihezi, China

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Abstract: The purpose of this study was to examine the repairing effect of KLD-12 polypeptide/TGF-β1 nanofiber gel combined with BMSCs in a rabbit intervertebral disc degeneration model. The rabbit models were divided into four groups after 2 weeks: KLD-12 polypeptide/TGF-β1/BMSCs gel (A), normal saline (B), KLD-12 polypeptide/BMSCs gel (C), and no treatment (D). X-ray examination revealed that the %DHI (disc height index) of group A increased from 4 weeks to 24 weeks and that these values were significantly higher than those of groups B and C (P<0.05). A similar trend was observed for the intensity of MRI T2-weighted signal. We observed newborn nucleus pulposus tissue in group A, grayish yellow fibrous hyperplasia in group B, white fibrous hyperplasia in group C; and translucent jelly-like tissue in group D. HE staining scores for groups A, B, C, and D were at levels 1-2, 4-5, 3-4, and 0, respectively. The nucleus pulposus cells in groups A and D were deep dyed with toluidine blue. The gray values for aggrecan and collagen II of group A showed slight increases from 4 weeks to 24 weeks, and these values were significantly higher than those of groups B and C (P<0.05). KLD-12 polypeptide/TGF-β1 nanofiber gel combined with BMSCs plays a role in the repair of degenerative intervertebral disc and promotes the regeneration of the nucleus pulposus tissue in the intervertebral disc in rabbits.

Keywords: KLD-12 polypeptide, transforming growth factor β1, mesenchymal stem cells, intervertebral disc degeneration

Introduction

Intervertebral disc degeneration is one of the main causes of low back pain and has a high prevalence [1]. Currently, the main methods of treatment include spinal fusion and nucleus pulposus removal. However, these treatment methods can only alleviate the symptoms, but cannot solve the fundamental problem of intervertebral disc degeneration [2]. A previous study showed that the main reason for the degeneration of intervertebral disc is the reduction of the water content in the extracellular matrix of the nucleus pulposus and nucleus pulposus cells and reduced amounts of extracellular matrix components such as protein polysaccharide and type II collagen [3]. There is no effective way to treat early stage degeneration of intervertebral discs and the residual cavity after the filling of the nucleus pulposus. Increased numbers of intervertebral disc cells can promote the formation of nucleus pulposus cells and the extracellular matrix to repair the structure and function of degenerated intervertebral discs.

Tissue engineering technology in the treatment of intervertebral disc degeneration shows a good application prospect [4-6]. In recent years, tissue engineering has provided new ideas for the treatment of intervertebral disc degeneration and for the filling the residual cavity after nucleus pulposus. The three core elements of tissue engineering are seed cells, biological materials, and growth factors. The simple application of cytokines cannot overcome the decrease in the number of intervertebral disc cells. BMSCs as seed cells have therefore been used in studies on intervertebral disc regeneration. BMSCs are relatively primitive mononuclear cells in the bone marrow, which are characterized by high proliferation and differentiation potential and low immunity. Yim RL et al. showed that BMSCs are
safe and effective for intervertebral disc regeneration [7]. Ryan JM et al. confirmed that BMSCs have the potential to self-renew and differentiate into a variety of functional cells, which can escape from recognition by homologous antigens [8]. Wei A et al. demonstrated that human BMSCs in rat intervertebral disk memory live for 6 weeks and express type II collagen and protein polysaccharide [9].

With regard to the biological material, the KLD-12 polypeptide is a novel self-assembling polypeptide, which has been shown to be able to assemble to form a complete structure of the hydrogel and the nanofiber network structure by itself [10, 11]. Oxalate is another option for the biological materials, but it has poor mechanical properties and cannot be degraded as a cell scaffold. In a previous study, Sun JH et al. showed that KLD-12 and its degradation products do not induce host immunologic rejection reactions or hemolytic reactions, do not affect the biological activity of intervertebral disc seed cells (BMSCs), and have good biocompatibility [11]. Bian Z et al. showed that KLD-12 peptide shows a sustained and slow release of TGF-β1 [12], and the KLD-12 peptide complex TGF-β1 may induce the differentiation of BMSCs into nucleus pulposus-like cells.

Growth factors such as transforming growth factor (TGF) play an important role in cell growth, differentiation, and secretion. Members of the TGF-β family, including the TGF-β1, TGF-β2, and TGF-β3 subtypes, which are encoded by different genes with different tissue distribution patterns, play a role in different biological activities in vivo [13]. TGF-β1 is the most widely used growth factor in intervertebral disc tissue engineering. Previously, Peng et al. showed that the level of TGF-β1 in degenerated intervertebral discs is significantly higher than that in normal intervertebral disc tissues [14]. Steck E found that TGF-β1 could induce BMSCs to synthesize type II collagen, protein polysaccharide, and other matrix components [15]. Risbud MV et al. showed that TGF-β1 could induce rat BMSCs to differentiate into nucleus pulposus-like cells in sodium alginate scaffolds under hypoxic conditions [16].

Currently, the application of tissue engineering for the treatment of intervertebral disc degeneration is still in the experimental stages. Most importantly, there is no uniform protocol for establishing animal models of intervertebral disc degeneration. Disc degeneration is mostly induced by fiber ring puncture, and a disc degeneration model can mimic discogenic pain, which has a high correlation with lumbar intervertebral disc degeneration [17].

In this study, we used KLD-12 polypeptide/TGF-β1 nanofiber gels combined with BMSCs in rabbit models of intervertebral disc degeneration. Our study shows the potential for the application of KLD-12 polypeptide/TGF-β1 nanofiber gels combined with BMSCs in the treatment of intervertebral disc degeneration.

Materials and methods

Animals

Procedures involving animals and their care were conducted in conformity with NIH guidelines (NIH Pub. No. 85-23, revised 1996) and was approved by the Animal Care and Use Committee of Shihezi University. Thirty-two healthy New Zealand white rabbits (age: 10 months, weight: 3±0.3 kg) were obtained from the Laboratory Animal Center of the School of Medicine, Shihezi University. The animals were divided into groups A, B, C, and D, with each group receiving different treatments.

Materials

KLD-12 polypeptide (AcN-KLDLKLDLKNH2, purity >98%) was purchased from Bio Engineering Ltd by Share Ltd. (Shanghai), TGF-1 was purchased from PeproTech (USA), RNA reverse transcription Kit was obtained from Thermo (USA), PCR primer and 5X TBE were obtained from Bio Engineering Ltd by Share Ltd. (Shanghai), agarose was obtained from Biowest (Spain), diethylpyrocarbonate (DEPC) was obtained from Sigma-Aldrich (Germany), and ISO alcohol was obtained from Rich Yu Fine Chemical Co., Ltd. (Tianjin).

Isolation, culture, and passage of rabbit BMSCs

The rabbits were anesthetized by injecting chlorm hydrate (10%) intravenously in the ear vein, and approximately 4 mL of bone marrow was extracted under sterile conditions from each of the rabbits. Cells were then extracted from the bone marrow samples using the density gradient method. LDMEM containing 10% FBS was used as the culture medium, and the cells were
cultured at 37°C in 5% CO₂. Half the medium was replaced with fresh medium after 24 h, and again after 48 h, and the whole medium was replaced every 3 days. When the cells reached 80-90% confluency, 0.25% trypsin was added for cell splitting.

**KLD-12 polypeptide/TGF-β1/BMSCs**

Freeze-dried powder of KLD-12 polypeptide (10 mg) was dissolved in 10% sucrose solution containing 5 μL of TGF-β1 (0.01 μg/μL) to prepare a 0.56% polypeptide (w/v) solution. One milliliter of the cell suspension containing 10 × 10⁶ cells/mL with 0.25% trypsin was prepared. The cell suspension was then mixed with the KLD-12 polypeptide/TGF-β1 solution and then subjected to oscillating mixing. The KLD-12 polypeptide/TGF-β1/BMSC suspension complex containing a final concentration of 0.5% KLD-12 polypeptide and 100 ng/mL TGF-β1 was thus prepared in vitro.

**Preparation of the rabbit model of lumbar intervertebral disc degeneration**

The rabbits were placed in the right lateral decubitus position, and the skin of the operation area was prepared and disinfected with iodophor and covered by the towel. A longitudinal incision was made on the skin on the left side (Figure 1A). The subcutaneous tissue and the muscle were then separated layer-by-layer through the retroperitoneal approach until the L3-4, L4-5, and L5-6 fiber rings were exposed (Figure 1B). Next, the fiber ring was punctured using a 16G puncture needle and the nucleus pulposus was aspirated (Figure 1C). The incision was then sutured layer by layer and bandaged by sterile dressing. Penicillin was injected intramuscularly after the operation to prevent infection. The dressing was changed regularly. The animals were placed in different cages and fed.

**Implantation of the KLD-12 polypeptide/TGF-β1/BMSCs**

After 2 weeks, the fiber rings were exposed and the graft was injected using a microsyringe. Group A received 40 μL of KLD-12 polypeptide/
Repair effect of KLD-12 polypeptide/TGF-β1 nanofiber gel and BMSCs in rabbits

TGF-β1/BMSCs gel, group B received only saline (40 μL), group C received 40 μL of the KLD-12 polypeptide/TGF-β1 gel, and group D did not undergo any treatment (control group).

*Plain radiographic imaging*

The vertebral body and the intervertebral space height were assessed using X-rays at 4, 8, 12, and 24 weeks after implantation. The intervertebral disc height index (DHI) was calculated as described previously by Lu et al. [18]; %DHI indicates the change in the height of the intervertebral disc caused due to the operation (%DHI=postoperative DHI/preoperative DHI×100%).

*MRI*

The animals were subjected to MRI at 4, 8, 12 and 24 weeks after implantation, and the signal of the T2-weighted images was measured.

*Macroscopic examination*

At 4, 8, 12 and 24 weeks after implantation, two New Zealand white rabbits were killed by sodium overdose. The lumbar spine was removed and fixed using 4% polyformaldehyde and incised at the upper and lower cartilage plate-bonding department. Next, the L3-4, L4-5, and L5-L6 intervertebral discs were removed and subjected to gross observation.

*HE staining*

The L3-4, L4-5, and L5-L6 intervertebral disc specimens were collected, fixed in 4% polyformaldehyde, dehydrated using a series of alcohols (anhydrous alcohol 1-2 grade), embedded in paraffin, and sliced in ~5-μm thick sections. The sections were then stained using HE stain, and histological grading was performed according to the standards described by Nishimura and Mochida in 1998 [19] (Table 1).

*Toluidine blue staining*

For toluidine blue staining, the intervertebral disc tissue specimens were fixed for 24 h in 10% polyformaldehyde and rapidly decalcified for 48 h. The tissue samples were then embedded in paraffin, sliced into ~5-μm thick sections, and stained with toluidine blue.

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Figure 2. X-ray at 12 weeks after transplantation. Group A: The disc height was restored; group B: The height of the intervertebral disc stenosis; group C: Intervertebral disc height recovery is not obvious; group D: The disc height shows no obvious change.
RT-PCR

The cryopreserved tissue samples were then taken out from the liquid nitrogen. Total RNA was extracted using Trizol reagent extraction and reverse transcribed RNA to cDNA using First Stand cDNA Synthesis. RT-PCR was then performed using the cDNA as the template. The primer sequences used for the PCR are shown in Table 2.

Statistical analysis

Experimental data was processed and analyzed using SPSS 17.0 statistical software. Variance analysis was used to compare the intervertebral disc height and the expression of aggrecan and Collagen II between groups A, B, C, and D after the normal state, and homogeneity of variance was satisfied using the levene test. The %DHI and the gray ratio of the PCR-amplified bands were shown as means ± standard deviation and were analyzed and compared through variance. P<0.05 was considered to indicate statistical significance.

Results

X-ray examination

The %DHI values in group A were 87.01±2.40%, 92.25±2.14%, 93.97±2.49%, and 94.98±1.91% at 4, 8, 12, and 24 weeks, respectively. The height of the intervertebral discs in group A showed obvious recovery at 4

Table 3. %DHI comparison of the four groups of animals at different time points (X±sDH)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>4 weeks</th>
<th>8 weeks</th>
<th>12 weeks</th>
<th>24 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>6</td>
<td>87.01±2.40</td>
<td>92.25±2.14</td>
<td>93.97±2.49</td>
<td>94.98±1.91</td>
</tr>
<tr>
<td>B</td>
<td>6</td>
<td>66.61±3.79a</td>
<td>61.65±3.92a</td>
<td>57.48±4.77a</td>
<td>48.74±5.46a</td>
</tr>
<tr>
<td>C</td>
<td>6</td>
<td>73.95±7.38ab</td>
<td>79.31±3.53ab</td>
<td>83.94±2.77ab</td>
<td>84.80±4.34ab</td>
</tr>
<tr>
<td>D</td>
<td>6</td>
<td>100.20±2.27abc</td>
<td>100.46±1.53abc</td>
<td>100.43±0.94abc</td>
<td>100.06±3.08abc</td>
</tr>
</tbody>
</table>

Note: compared with group A, *P<0.05; compared with group B, **P<0.05; compared with group C, ***P<0.05.
and 8 weeks and tended to be stable at 12 and 24 weeks. The height of the intervertebral discs in group B decreased significantly at 4 and 8 weeks and further showed a slight decrease at 12 and 24 weeks. In group C, no obvious recovery in the height of the intervertebral disc was observed. The intervertebral disc height in group D also did not show any obvious changes (Figures 2 and 3). Compared with group B, C, and D, the %DHI in group A showed a statistically significant difference (P<0.05) (Table 3).

**MRI**

The intensity of the MRI T2-weighted signal in group A at 4, 8, 12, and 24 weeks was 566.72±20.38, 703.71±30.01, 780.25±13.26, and 779.13±40.04, respectively. Group B showed a significantly low signal. The recovery of the signal in group C was not obvious, and there was no obvious change in the signal in group D at any time point (Figure 4). The difference between the signal in group A and that in groups B, C, and D at each of the time points showed a statistical significance (P<0.05) (Table 4).

**Table 4. T2-weighted image signals of the four groups of animals at different time points (s) (±s)**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>4 weeks</th>
<th>8 weeks</th>
<th>12 weeks</th>
<th>24 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>6</td>
<td>566.72±20.38</td>
<td>703.71±30.01</td>
<td>780.25±13.26</td>
<td>779.13±40.04</td>
</tr>
<tr>
<td>B</td>
<td>6</td>
<td>357.35±18.62</td>
<td>299.48±16.55</td>
<td>207.35±21.53</td>
<td>103.56±15.21</td>
</tr>
<tr>
<td>C</td>
<td>6</td>
<td>460.88±10.58</td>
<td>573.11±13.76</td>
<td>580.23±15.94</td>
<td>578.96±23.03</td>
</tr>
<tr>
<td>D</td>
<td>6</td>
<td>1115.53±4.39</td>
<td>1123.49±25.08</td>
<td>999.82±12.55</td>
<td>1105.45±23.46</td>
</tr>
</tbody>
</table>

Note: compared with group A, *P<0.05; compared with group B, **P<0.05; compared with group C, ***P<0.05.

Figure 4. MRI at 12 weeks after transplantation. Group A: Intervertebral disc signal slightly improved; group B: Intervertebral disc signal decreased significantly; group C: Intervertebral disc signal improvement is not obvious; group D: No change in the intervertebral disc signal.

**Gross specimen observation**

There was no obvious stenosis in the intervertebral space in group A, the nucleus pulposus was found in the central nucleus pulposus, and the structure of the peripheral fibers was normal (Figure 5A). In group B, the intervertebral...
space was narrow, and the central nucleus pulposus was absent (Figure 5B). In group C, there was a slight gap in the intervertebral space, and white fibrous tissue was found in the central nucleus pulposus area (Figure 5C). The intervertebral space in group D was not narrow (Figure 5D).

Toluidine blue staining showed that the nucleus pulposus in group A was deeply stained (Figure 8A) and that in group B showed loss of the nucleus pulposus and light local staining (Figure 8B). The nucleus pulposus in group C was moderately stained (Figure 8C), and that in group D was deeply stained (Figure 8D).

**HE staining**

In group A, the intervertebral disc structure was normal, and nucleus pulposus-like cells were seen in the central nucleus pulposus (Figure 6A); the peripheral fiber ring array layer was slightly disordered (Figure 7A). In group B, the intervertebral disc structure was obviously damaged, and the number of nucleus pulposus cells in the central nucleus pulposus area was significantly decreased; fibrous hyperplasia was also observed (Figure 6B). Moreover, the peripheral fiber ring structure was obviously disordered and fractured (Figure 7B). The intervertebral disc structure in group C was not clear, and fiber cartilage-like cells were observed in the central nucleus pulposus area (Figure 6C). The inner layer of the fiber ring structure was slightly disordered and the blood vessels showed local proliferation (Figure 7C). In group D, the nucleus pulposus area showed a large number of nucleus pulposus cells, and the intervertebral disc structure was normal (Figure 6D); the peripheral fiber ring was in a layer of neat arrangement (Figure 7D). The intervertebral disc degeneration in the four groups showed the following grades: group A: 1-2, group B: 4-5, group C: 3-4, and group D: -0.
Aggrecan mRNA was expressed in all the four groups (Figure 9). Semiquantitative results: The gray values for Aggrecan in group A at 4, 8, 12, and 24 weeks were 0.36±0.06, 0.37±0.05, 0.44±0.05, and 0.47±0.04, respectively. Aggrecan mRNA expression in group A was significantly higher (P<0.05) than that in groups B and C, but significantly lower than that in group D (P<0.05) at each time point (Table 5). Collagen II mRNA was also expressed in all the groups (Figure 10). Semiquantitative results: The gray values for collagen II in group A at 4, 8, 12, and 24 weeks were 0.37±0.01, 0.39±0.03, 0.37±0.02, and 0.39±0.01, respectively. Similar to the trend observed with aggrecan mRNA expression, collagen II mRNA expression in group A was significantly higher (P<0.05) than that in groups B and C, but significantly lower (P<0.05) than that in group D at all time points (Table 6).

Discussion

In this study, we constructed a bioactive KLD-12 polypeptide/TGF-β1 composite with BMSCs and studied its potential for use in the repair of degenerated intervertebral discs. We selected New Zealand white rabbits to establish the animal model of intervertebral disc degeneration and constructed the KLD-12 polypeptide/TGF-β1 nanofiber gel with biological activity. This nanofiber gel was combined with BMSCs in vitro and implanted into the rabbit degenerative intervertebral discs in situ. The KLD-12 polypeptide/TGF composite expressed type II collagen and protein polysaccharides. We found that implantation of KLD-12 polypeptide/TGF-β1/BMSCs could restore the height of the degenerated intervertebral discs. The signal strength of T2-weighted phases observed in the MRI also increased, which can be attributed to the indi-
rect reaction to the water content of the nucleus pulposus tissue [18]. Our MRI results showed that the T2-weighted signal value in the group treated with KLD-12/TGF-β1/BMSCs increased significantly, indicating that implantation of KLD-12/TGF-β1/BMSCs significantly promoted the degenerative intervertebral disc to secret extracellular matrix and to increase the water content of the discs. These results were consistent with those reported by Yang H et al. [19], where the T2-weighted signal intensity of the group treated with BMSCs was higher than that of the group treated with a TGF-β1 inhibitor. Gross observation and HE staining results showed that proteoglycan secretion from the degeneration intervertebral disc increased after implantation. RT-PCR revealed that the increase in collagen type II secretion maintained the height of the intervertebral discs as well as the histological features. The study by Yang H et al. [20] showed that the implantation of BMSCs and TGF-β1 inhibitors in rabbit models of intervertebral disc degeneration resulted in an increased number of spindle cells and increased type II collagen expression in the BMSC group, which indicated that intervertebral disc degeneration is an inflammatory disease. TGF-β1 is also a kind of immune suppression factor [21, 22]. NF-κB, which is a nuclear protein factor, has transcriptional regulatory activity and can induce immune response. I-κB is the inhibition unit of NF-κB. Yang H et al. showed that BMSCs delay disc degeneration by increasing TGF-β1 levels and reducing NF-κB levels [23]. TGF-β1 can also induce the expression of I-κB or inhibit the phosphorylation of NF-κB, thus downregulating the expression of NF-κB, which helps maintain the structure of intervertebral discs [24]. Thus, the implantation of KLD-12 polypeptide/TGF-β1 nanofiber gel comprising BMSCs significantly increased the amount of water and the protein content of the degenerated intervertebral discs in the early stages in our study, which helped repair and maintain the height of the intervertebral discs. It is also reasonable to believe that the nucleus pulposus-like tissues were regenerated and partially maintained the fibrous ring structure. This may be the regulatory mechanism underlying the action of TGF-β1 or BMSCs, which may be

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Gray scale ratio of aggrecan</th>
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<tbody>
<tr>
<td></td>
<td>4 weeks</td>
<td>8 weeks</td>
</tr>
<tr>
<td>A</td>
<td>9</td>
<td>0.36±0.06</td>
</tr>
<tr>
<td>B</td>
<td>9</td>
<td>0.21±0.03ab</td>
</tr>
<tr>
<td>C</td>
<td>9</td>
<td>0.27±0.04abc</td>
</tr>
<tr>
<td>D</td>
<td>9</td>
<td>1.47±0.04abcd</td>
</tr>
</tbody>
</table>

Note: compared with group A, *P<0.05; compared with group B, **P<0.05; compared with group C, ***P<0.05.
endogenous or exogenous and which promoted the functional recovery of the residual intervertebral disc cells in the nucleus pulposus tissue. Another possible explanation is that since BMSCs can differentiate into a variety of cells, in the microenvironment of the intervertebral discs, they differentiated into the nucleus pulposus cells and showed the nucleus pulposus cell phenotype and function induced by TGF-β1 and the microenvironment, which further promoted the repair of the degenerated intervertebral discs.

In summary, we constructed a KLD-12/TGF-β1 nanofiber gel with biological activity using tissue engineering and implanted it into degenerated intervertebral discs of rabbit models of intervertebral disc degeneration. We successfully combined the nanofiber gel with BMSCs and found that this combination had the capacity to repair and regenerate the function of the degenerated intervertebral discs. This experiment was limited to animal experiments; future studies should be focused on applying these data in humans. Our study suggests that a combination of KLD-12/TGF-β1 nanofiber gel and BMSCs can be effective for the treatment of intervertebral disc degeneration and to fill the residual cavity after nucleus pulposus.

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**Disclosure of conflict of interest**

None.

**Address correspondence to:** Jianhua Sun, Department of Orthopedics, The First Affiliated Hospital, Shihezi University College of Medicine, Shihezi 832008, China. Tel: +8618935706323; E-mail: 007sunjianhua@sina.com

**References**


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**Table 6. Comparison of Collagen II gray level of the four groups of animals at different time points**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>4 weeks</th>
<th>8 weeks</th>
<th>12 weeks</th>
<th>24 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>9</td>
<td>0.37±0.01</td>
<td>0.39±0.03</td>
<td>0.37±0.02</td>
<td>0.39±0.01</td>
</tr>
<tr>
<td>B</td>
<td>9</td>
<td>0.17±0.02</td>
<td>0.14±0.03</td>
<td>0.14±0.03</td>
<td>0.11±0.02</td>
</tr>
<tr>
<td>C</td>
<td>9</td>
<td>0.29±0.01</td>
<td>0.27±0.02</td>
<td>0.23±0.03</td>
<td>0.19±0.02</td>
</tr>
<tr>
<td>D</td>
<td>9</td>
<td>0.73±0.02</td>
<td>0.66±0.03</td>
<td>0.68±0.02</td>
<td>0.64±0.04</td>
</tr>
</tbody>
</table>

Note: compared with group A, aP<0.05; compared with group B, a,bP<0.05; compared with group C, a,b,cP<0.05.
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